BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

ELSEVIER

Molecular and Cellular Endocrinology 142 (1998) 35–39

MCE

A new tool for efficient transfection of dog and human thyrocytes in primary culture

N. Uyttersprot, S. Costagliola, F. Miot *

Interdisciplinary Research Institute, Free University of Brussels, 1070 Brussels, Belgium

Received 8 June 1998; accepted 9 June 1998

Abstract

The introduction of exogenous DNA into mammalian cells is commonly used to study the functions of gene products. However cells in primary culture are usually refractory to most transfection systems. Here we investigated the ability of a new lipid formulation, FuGENE™ 6 transfection reagent, to promote DNA uptake into dog and human thyroid cells in primary culture. Gene transfer was monitored by the expression of a Green Fluorescent Protein (GFP) reporter gene. We report that FuGENE 6 is particularly suited for the transfection of thyroid cells and does not interfere with their normal growth. Optimization of the experimental conditions, such as DNA amount, DNA/lipid ratio, cell density and incubation with the transfection mixture, was achieved by evaluating the percentage of GFP-expressing cells by FACS analysis. FuGENE 6 allowed us to obtain 8–15% thyrocytes expressing the reporter gene which represents an efficiency 100-fold superior to other transfection methods.

Keywords: Transfection; Lipid; Primary culture; Thyroid cells; GFP; FACS analysis

1. Introduction

Transfection has proved to be a potent method for the introduction of plasmidic DNA into eukaryotic cells. It is commonly used to overexpress genes for further characterization of the functions and properties of the encoded proteins. Several methods for DNA transfection have been developed during the past few decades. These are based on calcium phosphate-DNA coprecipitates (Graham and Van der Eb, 1973), polycations such as DEAE-dextran (Vaheri and Pagano, 1965) or polylysines (Zhou et al., 1991), electroporation (Neumann et al., 1982), cationic lipid-formed liposomes (Felgner et al., 1987), dendrimers (Kukowska Latallo et al., 1996) or scraping (Marshall and Leevers, 1995). Transfection of many established cell lines (COS-7, CHO-K1, ...) can be achieved by any of these methods, with high efficiency. However when dealing with cells in primary culture, most of these transfection systems are inefficient.

As in our laboratory the prevalent cellular model is the dog thyrocyte in primary culture (Roger et al., 1997), we were aware of this problem. Several transfection protocols (DEAE-dextran, calcium phosphate, liposomal reagents, ...) have already been tested on thyroid cells without any convincing result, i.e. yielding one transfected cell over 1000 (unpublished data). Therefore alternative methods such as microinjection (Dremier et al., 1997), retroviral vectors (Bond et al., 1994) or adenovirus-mediated cell infection (B. Pichon, personal communication) have been used to transfer plasmidic DNA into thyrocytes. However the availability of a powerful transfection method would still be of great interest because these methods are generally very easy to use and present several advantages over viral systems. Since good results were reported for the transfection of human primary foreskin keratinocytes using

* Corresponding author: Tel.: +32 2 5554152; fax: +32 2 5554655; e-mail address: fmiot@ulb.ac.be

0303-7207/98/$19.00
PII S0303-7207(98)00122-1

a new lipid formulation, the FuGENE™ 6 transfection reagent, we investigated, in this study, the efficiency of this product to transfect dog and human thyrocytes in primary culture. Because the transfection method described here was initially developed for the study of proteins involved in the cell cycle, the experimental conditions were optimized so that the procedure would not interfere with either the thyroid follicular structure or the proliferative properties of thyroid cells in primary culture.

2. Materials and methods

2.1. Primary culture of dog and human thyrocytes

Thyroid follicles were isolated from fresh tissue by collagenase digestion and differential centrifugation, as previously described (Roger et al., 1983; Roger and Dumont, 1987). Follicles were seeded in 35-mm Petri dishes at two different cell densities corresponding to 1 and 2×10^5 cells/dish, respectively.

2.2. Transfection procedure.

Transfections were carried out using the FuGENE™ 6 transfection reagent (Boehringer Mannheim) which is a proprietary blend of lipids in a non-liposomal formulation. As a reporter gene we used a plasmidic DNA encoding the Green Fluorescent Protein (GFP) under the control of the cytomegalovirus (CMV) immediate-early gene promoter. This construct has been generated by subcloning the open reading frame of GFP into the pcDNA3 expression vector (Invitrogen). Plasmidic DNA was prepared by means of Qiagen anion-exchange tips 500.

Depending on individual experiments, thyrocytes were transfected 2, 3, 4 or 5 days after seeding. For each transfection, FuGENE 6 was diluted in DMEM and mixed with plasmidic DNA, in the indicated amounts. Complex formation is a rapid process that requires only 15 min at room temperature to be completed. Thyrocytes were incubated with these DNA/FuGENE 6 complexes for 6, 24 or 48 h in the presence or absence of 10% foetal calf serum (FCS). Except for the 48 h incubation, the medium was then removed and replaced by fresh serum-free medium and thyrocytes were further cultured for 42 or 24 h, respectively.

3. Detection of GFP expression

The expression of Green Fluorescent Protein (GFP) in transfected thyrocytes was examined 48 h after transfection either by FACS analysis or by indirect immunofluorescence. FACS analysis was used to estimate the percentage of transfected cells by the detection of the natural fluorescence of GFP. Individual thyrocytes were released from follicles by a trypsin treatment (0.1%, 7 min, 37°C) and the fluorescence of 10000 cells was assayed by a FACScan flow cytofluorometer (Becton Dickinson). In some experiments the expression of GFP was also studied by indirect immunofluorescence: thyrocytes were fixed with 3% paraformaldehyde (10 min, 4°C), then permeabilized with 0.1% triton (10 min, room temperature). Because the natural fluorescence of GFP is very sensitive to this fixation step, GFP was detected by a polyclonal antibody (1/1000, Clontech) and fluorescein-conjugated anti-rabbit antibody (1/50, Amersham). Nuclei were counter-stained with 0.5 μg/ml propidium iodide.

4. Results and discussion

Specific experimental conditions of cell culture are required to maintain the integrity of thyroid function in vitro. Thyroid follicles are obtained by collagenase digestion of the thyroid tissue and separated from isolated cells by differential centrifugation. After seeding, follicles become adherent to the substrate and develop a cell monolayer by progressive spreading (Fig. 1, a and b). To investigate the ability of FuGENE 6 to transfect dog and human thyroid cells in primary culture, we used a plasmidic DNA encoding the Green Fluorescent Protein (GFP) under the control of the cytomegalovirus (CMV) immediate-early gene promoter as a reporter gene. As for other cell types, FuGENE 6 appeared to be non-toxic for dog and human thyrocytes and to transfect these cells very efficiently. After exposure to FuGENE 6 thyroid follicles remained healthy and continued to spread normally (Fig. 1b). Moreover we demonstrated elsewhere that these transfected thyroid cells retain the capacity to proliferate in response to mitogenic stimuli (manuscript in preparation). No cytotoxic effect was observed even if cells were cultured in the presence of FuGENE 6 for as much as 48 h. The expression of GFP in transfected thyrocytes was examined by indirect immunofluorescence using a polyclonal antibody against GFP because the natural fluorescence of GFP seems to be very sensitive to fixation conditions (Fig. 1c). Interestingly efficient transfection occurred almost exclusively on thyrocytes localized at the periphery of follicles. Cells confined more inside the follicular aggregates probably show little accessibility to the DNA/FuGENE 6 complexes.

We then decided to search for the optimal conditions for the FuGENE 6-mediated transfection of dog and human thyroid cells in primary culture. For each experiment the efficiency of the method (i.e. the percentage of GFP-expressing cells) was evaluated 48 h after trans-

fection by FACS analysis on 10000 individual thyrocytes released from follicles by a trypsin treatment. Fig. 2 illustrates the typical distribution of fluorescence measured by the flow cytofluorometer. When thyrocytes were transfected with the wild-type expression vector, the distribution of fluorescence corresponded to the intrinsic fluorescence of these cells (Fig. 2a). By contrast, when thyrocytes were transfected with the GFP-encoding expression vector, a significant shift of fluorescence was observed for a proportion of the cell population (Fig. 2b). In this experiment about 16% of thyrocytes showed a 100 to 1000-fold increase in the intensity of fluorescence representing the natural fluorescence of overexpressed GFP. Using this method we examined the effect of varying several parameters on the efficiency of FuGENE 6-based gene transfer into thyrocytes. All the results presented below were obtained on both dog and human thyroid cells.

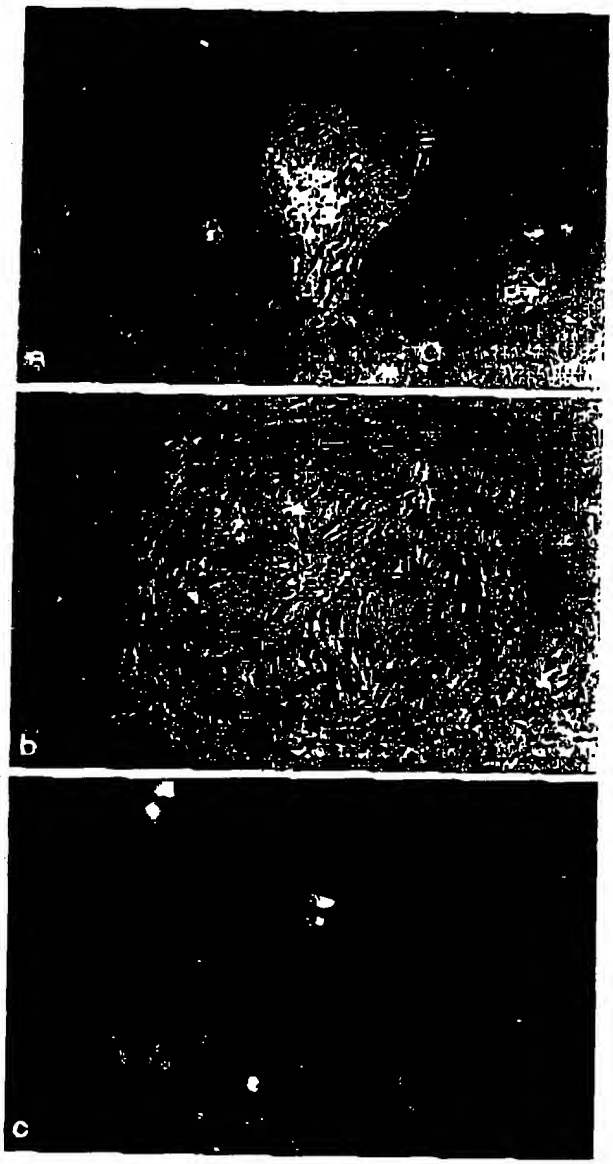

Fig. 1. Morphology of thyroid cells in primary culture. (a, b) Phase contrast microscopy of a dog thyroid follicle. Three days after seeding (a), cells were transfected with FuGENE 6 using a 2/6 ratio. The incubation with the transfection mixture was carried out in 10% FCS for 48 h. Two days after transfection, the follicle looks healthy and is now fully spread (b). (c) Detection of GFP expression in transfected dog thyrocytes by indirect immunofluorescence, 48 h after transfection. The fluorescence is uniformly distributed along the cytoplasm of transfected cells. The intensity of fluorescence varies among transfected cells indicating various level of expression of the reporter gene.

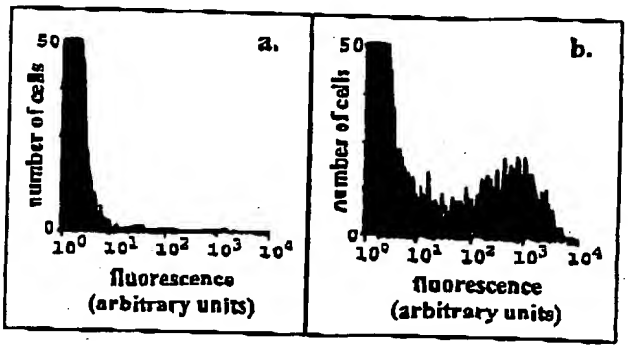

Fig. 2. FACS analysis of thyroid cells after transfection. For this experiment dog thyrocytes were transfected 3 days after seeding with either the wild-type pcDNA3 expression vector (a) or the GFP-encoding pcDNA3 expression vector (b). Transfection was achieved using 2 μg of plasmidic DNA complexed to 6 μl of FuGENE 6 and cells were exposed to this transfection mixture for the first 6 h in the presence of 10% FCS. FACS analysis was performed 48 h after transfection. (a) The distribution of fluorescence corresponds to the intrinsic fluorescence of thyrocytes in primary culture. (b) The first peak of fluorescence is superposable to the one obtained with the wild-type expression vector or with untransfected cells while the second represents the GFP-expressing cells.

In cationic lipid-mediated transfections, one important factor controlling the efficiency is the DNA/lipid ratio. A complex is formed by interactions of negative charges on DNA molecules with positively-charged lipids. But this complex has to keep a net positive charge for maximal interactions with plasma membranes. Therefore we first examined the efficiency of transfection using various DNA/FuGENE 6 ratios and established that the optimal ratio was 1 to 3 (μg of DNA/μl of FuGENE 6). Indeed a 2-fold increase of the DNA content complexed with a constant amount of FuGENE 6 (corresponding to a 2/3 ratio) dramatically inhibited the transfection process (data not shown). However for this optimal 1/3 ratio, higher percentages of transfected cells (up to 19%) could be further obtained by increasing proportionally DNA and FuGENE 6 quantities (Fig. 3a). Importantly, exposure of thyrocytes to these larger amounts of FuGENE 6 (up to 9 μl) did not result in any obvious cytotoxic effect.

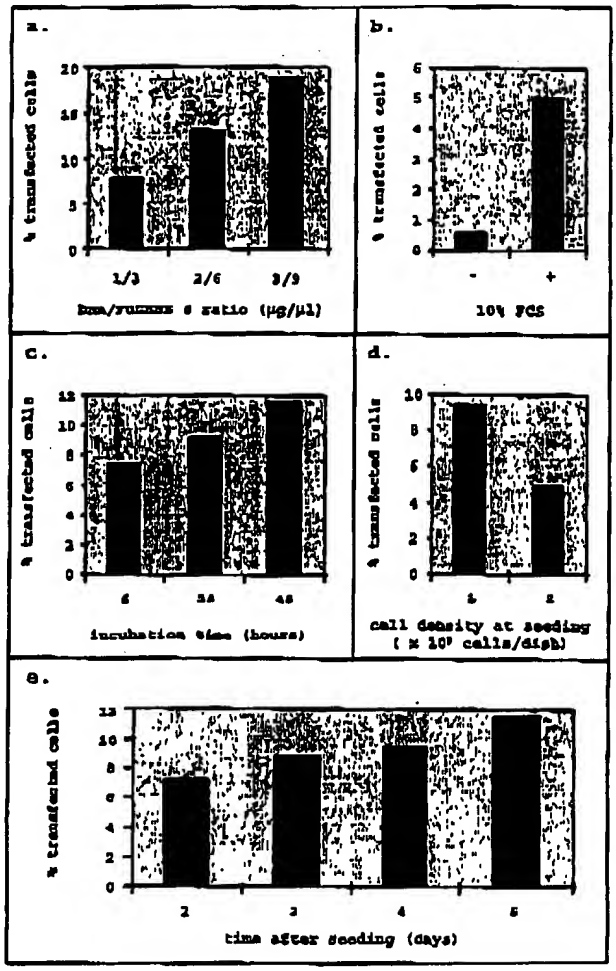

Fig. 3. Optimization of the FuGENE 6-mediated transfection of thyroid cells in primary culture. For these individual experiments we used the same general protocol (transfection of the GFP reporter gene), varying only one factor each time. The efficiency of the method was measured by the percentage of transfected cells as estimated by FACS analysis 48 h after transfection. (a) Human thyrocytes were seeded at 100000 cells/dish and transfected 4 days later with different quantities of DNA (μg). The amount of FuGENE 6 (μl) was increased proportionally so that a constant 1/3 ratio of DNA to FuGENE 6 was used. The incubation with the transfection mixture was carried out in 10% FCS for 48 h. (b) Human thyrocytes were seeded at 100000 cells/dish and transfected 3 days later using a DNA/FuGENE 6 ratio of 2/6. The incubation with the transfection mixture was performed for 6 h in the culture medium containing or not 10% FCS. (c) Human thyrocytes were seeded at 100000 cells/dish and transfected 5 days later using a DNA/FuGENE 6 ratio of 2/6. The incubation with the transfection mixture was performed in 10% FCS for 6, 24 or 48 h. (d) Human thyrocytes were seeded at 100,000 or 200,000 cells/dish and transfected 4 days later using a DNA/FuGENE 6 ratio of 2/6. The incubation with the transfection mixture was carried out in 10% FCS for 48 h. (e) Human thyrocytes were seeded at 100000 cells/dish. Transfection was carried out at different times of the culture using a DNA/FuGENE 6 ratio of 2/6. The incubation with the transfection mixture was carried out in 10% FCS for 48 h.

It has been known for a long time that proliferating cells, such as transformed cell lines, can be transfected more easily than quiescent cells (Nicolau and Sene, 1982; Strain et al., 1985; Ray and Gage, 1992; Harrison et al., 1995). The breakdown of the nuclear membrane during the transition from the G_2 phase to mitosis could account for this observation (Nicolau and Sene, 1982). However this cannot be the sole explanation since an increase in the transfection efficiency was also obtained on EGF-stimulated hepatocytes before the onset of DNA synthesis (Somasundaram et al., 1992). In classical conditions of culture, dog and human thyrocytes are maintained in a defined serum-free medium (Roger et al., 1983; Roger and Dumont, 1987). Such thyroid cells are quiescent but can be directed into the cell cycle by different stimuli, including foetal calf serum (FCS). We thus asked whether a serum treatment would facilitate the FuGENE 6-mediated transfection of thyrocytes. As serum greatly inhibits interactions between plasmidic DNA and cationic lipids (Yang and Huang, 1997), DNA and FuGENE 6 were first mixed in a serum-free medium (either DMEM, PBS or KRH). Thyrocytes were then exposed to the DNA/FuGENE 6 complexes in the complete medium containing 10% FCS for various times. After this incubation, cells were washed free of FuGENE 6 and serum and cultured further in a serum-free medium. Gene transfer appeared to be 10-fold more effective in the presence of serum compared to the transfections performed in a serum-free medium (Fig. 3b). Because this effect could already be observed after a 6 h exposure to serum, we cannot be sure it is a consequence of the entry of thyrocytes into the cell cycle. This effect could be mediated more directly by one of the components of serum. Furthermore the increase of transfection efficiency was even more pronounced for the longer incubations reaching 12% positive cells (Fig. 3c). However in this case it probably reflects the long-term action of FuGENE 6 rather than an effect of serum because the same difference in efficiency between these incubation times could also be obtained in the absence of serum (data not shown).

Another important point that has to be considered is the cell density of the culture at the time of transfection. In our cellular model, the available cell surface area for interactions with DNA/FuGENE 6 complexes is a function of the cell density at seeding as well as of the spreading of thyroid follicles (i.e. the age of the culture). Transfection of thyrocytes by FuGENE 6 appeared to be most efficient when follicles were seeded at a lower density (Fig. 3d). Increased percentage of positive cells was also obtained when transfection was performed on fully-spread thyroid follicles (Fig. 3e). Indeed the microscopic examination revealed that for this culture maximal spreading of follicles was reached after 5 days of culture which correlates with the highest

efficiency of transfection (12%). However it has to be noticed that progressive spreading resulting in contacts between distinct follicles will lower rather than increase the uptake of DNA. This problem is frequently encountered with larger thyroid follicles and accounts for the variability of transfection efficiency observed from one preparation of thyroid cells to another.

In conclusion, the FuGENE™ 6 transfection reagent is a powerful tool for the transfection of dog and human thyroid cells in primary culture. We defined specific conditions of transfection that yield maximal efficiency. Thyroid follicles are seeded in 35-mm Petri dishes so that approximately 100000 cells are cultured per dish. Three to 4 days later, corresponding to the time where follicles present maximal spreading, thyrocytes are transfected using 2 μg of plasmidic DNA complexed to 6 μl of FuGENE 6. Incubation of cells with this transfection mixture is performed in a 10% FCS-supplemented medium for 48 h. However if serum is suspected to induce any side effect on the functional assay (excessive proliferation, inhibition of differentiation, ...) the incubation time in the presence of serum can be reduced to 6 h. For transient transfection using this method and a reporter gene whose transcription is driven by the CMV promoter, we obtained the best results when the functional assay was carried out 48 h after transfection. This protocol has now been used on many primary cultures of dog and human thyrocytes ($n > 10$) yielding 8 to 15% transfected cells. FuGENE 6-mediated transfection is 100 to 200-fold more effective on thyroid cells than any other previously tested method and will become the prevalent tool for many applications.

Acknowledgements

This work was supported by grants from the Ministère de la Politique Scientifique (PAI), the Association contre le cancer and the Association sportive contre le cancer. UN is a fellow of Télévie (Fonds National de la Recherche Scientifique).

References

Bond, J.A., Wyllie, F.S., Rowson, J., Radulescu, A., Wynford-Thomas, D., 1994. In vitro reconstruction of tumour initiation in a human epithelium. Oncogene 9, 281–290.

Dremier, S., Pohl, V., Poteet Smith, C., Roger, P.P., Corbin, J., Doskeland, S.O., Dumont, J.E., Maenhaut, C., 1997. Activation of cyclic AMP-dependent kinase is required but may not be sufficient to mimic cyclic AMP-dependent DNA synthesis and thyroglobulin expression in dog thyroid cells. Mol. Cell Biol. 17, 6717–6726.

Felgner, P.L., Gadek, T.R., Holm, M., Roman, R., Chan, H.W., Wenz, M., Northrop, J.P., Ringold, G.M., Danielsen, M., 1987. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. Proc. Natl. Acad. Sci. USA 84, 7413–7417.

Graham, F.L., Van der Eb, A.J., 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology 52, 456–467.

Harrison, G.S., Wang, Y., Tomczak, J., Hogan, C., Shpall, E.J., Curiel, T.J., Felgner, P.L., 1995. Optimization of gene transfer using cationic lipids in cell lines and primary human CD4+ and CD34+ hematopoietic cells. Biotechniques 19, 816–823.

Kukowska Latallo, J.F., Bielinska, A.U., Johnson, J., Spindler, R., Tomalia, D.A., Baker, J.R., 1996. Efficient transfer of genetic material into mammalian cells using Starburst polyamidoamine dendrimers. Proc. Natl. Acad. Sci. USA 93, 4897–4902.

Marshall, C.J., Leevers, S.J., 1995. Mitogen-activated protein kinase activation by scrape loading of p21ras. Methods Enzymol. 255, 273–279.

Neumann, E., Schaefer Ridder, M., Wang, Y., Hofschneider, P.H., 1982. Gene transfer into mouse lyoma cells by electroporation in high electric fields. EMBO J. 1, 841–845.

Nicolau, C., Sene, C., 1982. Liposome-mediated DNA transfer in eukaryotic cells. Dependence of the transfer efficiency upon the type of liposomes used and the host cell cycle stage. Biochim. Biophys. Acta 721, 185–190.

Ray, J., Gage, F.H., 1992. Gene transfer into established and primary fibroblast cell lines: comparison of transfection methods and promoters. Biotechniques 13, 598–603.

Roger, P.P., Dumont, J.E., 1987. Thyrotropin is a potent growth factor for normal human thyroid cells in primary culture. Biochem. Biophys. Res. Commun. 149, 707–711.

Roger, P.P., Servais, P., Dumont, J.E., 1983. Stimulation by thyrotropin and cyclic AMP of the proliferation of quiescent canine thyroid cells cultured in a defined medium containing insulin. FEBS Lett. 157, 323–329.

Roger, P.P., Christophe, D., Dumont, J.E., Pirson, I., 1997. The dog thyroid primary culture system: a model of the regulation of function, growth and differentiation expression by cAMP and other well-defined signaling cascades. Eur. J. Endocrinol. 137, 579–598.

Somasundaram, C., Tournier, I., Feldmann, G., Bernuau, D., 1992. Increased efficiency of gene transfection in primary cultures of adult rat hepatocytes stimulated to proliferate: a comparative study using the lipofection and the calcium phosphate precipitate methods. Cell Biol. Int. Rep. 16, 653–662.

Strain, A.J., Wallace, W.A., Wyllie, A.H., 1985. Enhancement of DNA-mediated gene transfer by high-Mr carrier DNA in synchronized CV-1 cells. Biochem. J. 225, 529–533.

Vaheri, A., Pagano, J.S., 1965. Infectious poliovirus RNA: a sensitive method of assay. Virology 27, 434–436.

Yang, J.P., Huang, L., 1997. Overcoming the inhibitory effect of serum on lipofection by increasing the charge ratio of cationic liposome to DNA. Gene Ther. 4, 950–960.

Zhou, X.H., Klibanov, A.L., Huang, L., 1991. Lipophilic polylysines mediate efficient DNA transfection in mammalian cells. Biochim. Biophys. Acta 1065, 8–14.